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Development of a Multiple-Locus Variable-Number Tandem-Repeat Typing Scheme for Genetic Fingerprinting of *Burkholderia cenocepacia* and Application to Nationwide Epidemiological Analysis

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Organisms of the *Burkholderia cepacia* complex are especially important pathogens in cystic fibrosis (CF), with a propensity for patient-to-patient spread and long-term respiratory colonization. *B. cenocepacia* and *Burkholderia multivorans* account for the majority of infections in CF, and major epidemic clones have been recognized throughout the world. The aim of the present study was to develop and evaluate a multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) scheme for *B. cenocepacia*. Potential VNTR loci were identified upon analysis of the annotated genome sequences of *B. cenocepacia* strains AU1054, J2315, and MCO-3, and 10 of them were selected on the basis of polymorphisms and size. A collection of 100 *B. cenocepacia* strains, including epidemiologically related and unrelated strains, as well as representatives of the major epidemic lineages, was used to evaluate typeability, epidemiological concordance, and the discriminatory power of MLVA-10 compared with those of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Longitudinal stability was assessed by testing 39 successive isolates from 14 patients. Typeability ranged from 0.91 to 1, except for that of one marker, which was not amplified in 53% of the *B. cenocepacia* IIIA strains. The MLVA types were shown to be stable in chronically colonized patients and within outbreak-related strains, with excellent epidemiological concordance. Epidemic and/or globally distributed lineages (epidemic Edinburgh-Toronto electrophoretic type 12 [ET-12], sequence type 32 [ST-32], ST-122, ST-234, and ST-241) were successfully identified. Conversely, the discriminatory power of MLVA was lower than that of PFGE or MLST, although PFGE variations within the epidemic lineages sometimes masked their genetic relatedness. In conclusion, MLVA represents a promising cost-effective first-line tool in *B. cenocepacia* surveillance.

The *Burkholderia cepacia* complex (BCC), which includes 18 closely related species, is mainly involved in pulmonary infections in patients with cystic fibrosis (CF), although it is also occasionally recovered in nosocomial infections and/or from immunocompromised hosts. BCC infection in CF patients is associated with accelerated decline of lung function, cepacia syndrome, and poor posttransplantation outcome. The most frequently recovered species of the BCC are *B. cenocepacia* and *Burkholderia multivorans*, which have been found to be responsible for large outbreaks among CF patients, prompting the implementation of infection control measures and epidemiological surveillance. *B. cenocepacia* consists of four *recA* subgroups: IIIA, which includes the Edinburgh-Toronto electrophoretic type 12 (ET-12) epidemic lineage (in Canada and the United Kingdom) (1); IIIB, which includes the PHDC epidemic lineage (in the United States) (2) and the Midwest clone (3); IIIC, which is exclusively environmental; and IIID, which was reported to be responsible for 50% of the BCC infections in an Italian CF center (4).

Putative transmissibility markers have been identified within *B. cenocepacia* epidemic strains. The *cblA* pilin gene is characteristic of ET-12 strains (5). The *B. cepacia* epidemic strain marker (BCESM), which is part of a pathogenicity island, was identified in ET-12 strains and in other epidemic lineages but also in unique clinical or environmental strains (6, 7), whereas the PHDC lineage is BCESM negative. Finally, the IS*1363* insertion sequence was demonstrated to be characteristic of the PHDC and ET-12 lineages (8). Thus, some epidemic lineages are associated with genetic markers, but none of these proved to be universal, and epidemiological surveillance still requires genotyping studies.

Several typing methods have been developed to trace BCC organisms. Among them, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are currently considered the gold standard methods for epidemiological typing and population genetics studies. PFGE has been extensively used to characterize outbreaks but is a rather laborious technique that requires 9

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days to generate typing results. Another limitation of PFGE typing is the difficulty in comparing the fingerprints between laboratories, especially when different protocols and similarity thresholds for clonal delineation are used. MLST offers the advantage of being a truly portable method that allows the generation of coded types and easily accessible databases. However, MLST costs are too high for routine use in the clinical microbiology laboratory or for the characterization of large numbers of BCC strains in national surveillance programs. Additional typing methods combining good interlaboratory reproducibility with high discrimination, meaningful epidemiological inference, and low cost are still needed.

Tandem repeats (TR) consist of consecutive occurrences of a more or less identical DNA repeat unit. They are found in the chromosomes of most organisms, from prokaryotes to eukaryotes, and some TR show intraspecies polymorphisms. Although the biological function and evolution mechanism(s) of these loci with variable-number tandem repeats (VNTRs) are not yet fully understood, they have found several practical applications in epidemiological typing. Multilocus VNTR analysis (MLVA) is one of the most promising techniques for tracing the epidemiology of bacteria and has become the reference typing method in several bacterial species (9–13). In a typical MLVA assay, a predefined number of VNTRs is analyzed by PCR amplification, followed by electrophoresis to estimate the size of the PCR products. A code reflecting the number of repeats at each locus can be determined for individual strains. Therefore, MLVA is a cost-effective typing method that provides portable results, with the MLVA codes consisting of an unequivocal string of numbers that can easily be exchanged between laboratories and made accessible via internet databases (12). MLVA codes can also be deduced from whole-genome sequence data (14). An MLVA scheme has been developed to type *Burkholderia pseudomallei* (15, 16).

The aim of this study was to establish an MLVA scheme for *B. cenocepacia*. For this purpose, we searched for the presence of shared VNTR markers in the complete genome sequences of four *B. cenocepacia* strains and set up a standard MLVA protocol based on simple PCR and agarose gel electrophoresis. The overall performances of the MLVA method were assessed in a study of a selected collection of *B. cenocepacia* strains and compared with those of reference typing methods, including PFGE and MLST.

MATERIALS AND METHODS

Bacterial strains. A total of 109 strains were used in the present study, which included 25 reference strains and 84 strains from the French Observatoire *Burkholderia cepacia* (OBC) collection. The 25 reference strain panel included seven *B. cenocepacia* IIIA strains (J2315, LMG18827, LMG18826, LMG18863, LMG6981, LMG18828, and LMG6986), nine *B. cenocepacia* IIIB strains (AU1054, LMG16659, LMG18829, LMG18830, LMG13011, LMG14271, LMG14274, LMG14276, and LMG16654), eight *B. cenocepacia* IIIC strains (LS2.4, Aus26, Aus12, Aus31, Aus32, Aus34, Aus13, and WS11.7, referred to in the LMG collection as LMG19233, LMG19247, LMG19243, LMG19244, LMG19248, LMG19242, LMG19240, and LMG19232, respectively), and one *B. cenocepacia* IIID strain (LMG21461). Strain AU1054 (PHDC lineage) was kindly provided by John LiPuma, University of Michigan, Ann Arbor, MI, USA, and strain J2315 (ET-12 lineage) by Catherine Doherty, University of Edinburgh, Edinburgh, Great Britain; other clinical strains were obtained from the LMG collection, Ghent, Belgium. The environmental *B. cenocepacia* IIIC strains were kindly provided by Jacques Balandreau, UMR 5557 CNRS, Université Claude Bernard, Lyon, France. Nine reference strains were part of the diagnostically and experimentally useful panel of strains of the BCC (17). The panel of 84 nonredundant clinical strains from the OBC collection included epidemiologically related and unrelated strains (Table 1; see also Table S2 in the supplemental material) and comprised 72 CF strains from 20 CF centers, as well as 12 non-CF strains. These 84 strains had been tested with the species-specific *recA*-based PCRs described by Mahenthiralingam et al. (18), and 46 were assigned to *B. cenocepacia* IIIA and 37 to *B. cenocepacia* IIIB. One strain was species-specific PCR negative and was subsequently identified as *B. cenocepacia* IIID by means of *recA* sequencing.

Also, 39 clinical isolates recovered from 14 patients (two to four successive isolates per patient sampled over a mean period of 4.7 years) were included to assess the *in vivo* stability of the MLVA results (longitudinal stability panel; see Table S1 in the supplemental material).

Selection of VNTR markers. The annotated genome sequences of strain AU1054 (GenBank accession no. NC_008060, NC_008061, and NC_008062), J2315 (NC_011000, NC_011001, and NC_011002), and strain MCO-3 (NC_010508, NC_010512, and NC_010515) were inspected for the presence of potential VNTR loci by using the strain comparison tool available at the Microorganisms Tandem Repeats Database (http://tandemrepeat.u-psud.fr/). Ten VNTRs were selected on the basis of polymorphisms and repeat size (Table 2). The full locus names include the following allele calling convention: repeat unit size, expected PCR product size with the indicated primers in the first published complete genome sequence (J2315), and corresponding repeat unit number assigned by convention (for example, BcenM01_49bp_399bp_4U). Abbreviated names (BcenM01) are used here for simplification.

Nomenclature and description of MLVA profiles. The expected amplicon size, repeat length, and number of repetitions were determined in reference genomes of the AU1054, MC0-3, and J2315 strains available at the National Center for Biotechnology Information (see http://www.ncbi.nlm.nih.gov/sites/genome), using the PCR primer BLAST tool from the Microorganisms Tandem Repeats Database and an *in silico* MLVA typing tool (see http://mlva.u-psud.fr). The number of repeats was calculated according to an excess approximation in order to consider also the non-integer number of repeats in the allele counts, as explained in Vergnaud and Pourcel (10, 13). The length of the individual repeats within some of the tandem repeats showed variations (Table 2), but repeat copy numbers were unambiguously derived from size estimates, mostly due to large repeat units and moderate repeat copy numbers. The allelic profile was defined as the number of repeats at each VNTR locus included in the MLVA scheme. When no amplification was repeatedly observed at a given locus, the value "0" was assigned to the corresponding allele.

Template DNA for PCRs. Bacterial thermolysates were prepared from overnight cultures using a rapid procedure; four colonies were suspended in 200 μl of sterile water, incubated 10 min at 95°C, frozen at −20°C, and thawed. The supernatant was recovered after centrifugation and stored at −20°C.

PCR amplification of VNTR loci. The primers used are described in Table 2. PCR was performed in a reaction volume of 25 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 1 M betaine (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 1 U of Taq DNA polymerase (Eurobio, Les Ulis, France), 200 μM each deoxynucleoside triphosphate (Eurobio), 0.2 μM each primer (Eurofins MWG Operon, Les Ulis, France), and 2.5 μl of DNA template. Amplification was carried out using a VWR Doppio thermal cycler, under the following conditions: initial denaturation at 96°C for 4 min, 30 cycles of denaturation at 96°C for 30 s, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. One microliter of PCR product was separated in 2% (for BcenM01, BcenM02, BcenM03, BcenM04, BcenM07, and BcenM10), 1.5% (for BcenM05 and BcenM09), or 1% (BcenM06 and BcenM08) (wt/vol) agarose gels (Resophor; Eurobio) in Tris-borate-EDTA (TBE) buffer (Euromedex, Souffelweyersheim, France). The PCR products from

TABLE 1 Characteristics and genotypes of the epidemiologically related panel of strains used for the evaluation of *in vivo* transversal stability and of the epidemiological concordance of MLVA ($n = 39$)

Strain[a]	*recA* subgroup lineage	Source	Location	PCR ribotype	PFGE	MLVA profile[c]	MLVA type	IS*1363*	MLST results[d]							
									atpD	*gltB*	*gyrB*	*recA*	*lepA*	*phaC*	*trpB*	ST
French outbreak-related strains ($n = 25$)																
OBC01_52	IIIA French type 3 ($n = 8$)[b]	CF patient	France (Bretagne)	A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1		16	11	10	14	11	6	79	32
OBC02_21		CF patient		A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1										
OBC15_34		CF patient		A	BE	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1		16	11	10	14	11	6	79	32
OBC21-17		CF patient		A	BD	3, 0, 2, 3, 2, 1, 2, 9, 0, 3	A24									
OBC02_79		CF patient	France (PACA)	A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1									
OBC03_03		CF patient		A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1									
OBC01_72		CF patient	France (Lorraine)	A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1									
OBC01_01		CF patient		A	BD	3, 3, 2, 3, 2, 1, 2, 9, 0, 3	A1									
OBC05_12	IIIB French type 11 ($n = 9$)[b]	CF patient	France (PACA)	D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−	16	16	266	191	68	41	13	279
OBC05_36		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC17_29		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 0	B21	−								
OBC01_06		CF patient	France (Alsace/Lorraine)	D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC08_67		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC07_33		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC05_74		CF patient	France (Bretagne)	D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC06_46		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC06_25		CF patient		D	C	2, 6, 4, 2, 3, 4, 2, 9, 4, 6	B2	−								
OBC12_32	IIIB French type 2 ($n = 8$)[b]	CF patient	France (Auvergne)	L	DZ	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+	23	134	57	15	93	8	14	122
OBC07_30		CF patient		L	DX	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+	23	134	57	15	93	8	14	122
OBC12_36		CF patient		L	DX	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+								
OBC12_38		CF patient		L	DX	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+								

OBC12_44		CF patient		L	DX	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+							
OBC01_38		CF patient		L	M	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+	23	134	57	15	93	8	14	122
OBC12_40		CF patient		L	DY	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+	23	134	57	15	93	8	14	122
OBC12_39		CF patient		L	DY	3, 5, 4, 4, 3, 4, 3, 8, 4, 5	B1	+							
French epidemiologically related strains ($n = 6$)															
OBC05_31	IIIA	CF patient	France (Midi-Pyrénées)	E	GN	4, 3, 2, 3, 2, 1, 2, 8, 0, 3	A3		15	11	182	14	11	6	147	234
OBC03_16		CF patient		E	FN	4, 3, 2, 3, 2, 1, 2, 8, 0, 3	A3		15	11	182	14	11	185 (2)	147	869
OBC22_78	IIIA	CF patient	France (PACA)	FK	HB	4, 3, 3, 0, 2, 1, 2, 8, 2, 3	A26		16	11	286	14	11	6	147	757
OBC22_79		CF patient	France (Languedoc)	FK	HB	4, 3, 3, 3, 2, 1, 2, 8, 2, 3	A4		16	11	286	14	11	6	147	757
OBC22_59	IIIA	CF patient	France (Bretagne)	A	GO	3, 3, 5, 3, 2, 1, 2, 9, 3, 3	A5		130	11	590	14	11	6	147	844
OBC23_05		Non-CF patient		A	GO	3, 3, 5, 3, 2, 1, 2, 9, 3, 3	A5									
ET-12 lineage ($n = 4$)															
J2315	IIIA	CF patient	UK			4, 3, 1, 3, 2, 1, 2, 9, 2, 3	A6		15	11	9	14	11	6	12	28
LMG18827		CF patient	Canada			4, 3, 1, 3, 2, 1, 2, 9, 2, 3	A6		15	11	9	14	11	88 (8)	12	29
LMG18826		CF patient	Canada			4, 3, 1, 3, 2, 1, 2, 9, 2, 3	A6		15	11	9	14	11	6	79 (2)	31
LMG18863		CF patient	Canada			4, 3, 1, 3, 2, 1, 2, 9, 2, 3	A6		21 (5)	11	13 (3)	14	11	6	12	30
Environmental IIIC strains ($n = 4$)															
LMG19244	IIIC	Wheat pasture	Australia (Kapunda)			1, 3, 4, 3, 3, 4, 0, 0, 7, 1	C1		62	105	111	47	33	36	93	118
LMG19248		Wheat pasture				1, 3, 4, 3, 3, 4, 0, 0, 7, 1	C1		62	105	111	47	33	36	93	118
LMG19242		Wheat pasture				1, 3, 4, 3, 3, 4, 0, 0, 7, 1	C1		62	105	111	47	33	36	93	118
LMG19243		Wheat pasture				1, 3, 4, 3, 3, 4, 0, 0, 7, 1	C1		62	105	111	47	33	36	93	118

[a] OBC, Observatoire *B. cepacia* collection; LMG, Laboratorium voor Microbiologie Gent, Belgium.
[b] As referred to by Segonds et al. (20).
[c] Repeat copy numbers at loci M01, M02, M03, M04, M05, M06, M07, M08, M09, and M10, respectively; 0, no amplification product.
[d] MLST results for reference strains, originating from the *B. cepacia* complex MLST website (http://pubmlst.org/bcc/) developed by Keith Jolley and sited at the University of Oxford (23), are underlined; ST, sequence type; the number of nucleotide differences between the MLST alleles in strains sharing the same MLVA profiles and belonging to different STs are in parentheses.

TABLE 2 Characteristics and performances of the 10 selected VNTR loci

VNTR locus	Size of repeat unit (bp)	Product size (bp) (no. of repeats) in strain:			Primer sequence (5′ to 3′)[a]	MLVA-10 analysis of:								
						100 *B. cenocepacia* strains						60 epidemiologically unrelated IIIA and IIIB strains (HGDI values [95% CI]) for[b]:		
						Repeat range (no. of alleles)	No. (%) of amplification failures for:							
		MC0-3	J2315	AU1054			IIIA ($n = 49$)	IIIB ($n = 41$)	IIIC ($n = 8$)	IIID ($n = 2$)	Total ($n = 100$)	IIIA ($n = 36$)	IIIB ($n = 24$)	Total ($n = 60$)
BcenM01_49bp_399bp_4U	49	308 (2)	399 (4)	357 (3)	F: GTAGCGCAGCTCGTAGTCGT; R: CTACTGGATGGACCGGTGAG	0–4 (5)	0 (0)	2 (4.8)	0 (0)	0 (0)	2 (2)	0.53 (0.47–0.60)	0.59 (0.43–0.75)	0.69 (0.65–0.72)
BcenM02_15bp_207bp_3U	15	225 (4)	207 (3)	240 (5)	F: TAAGCCACGAAAGCCCGGCAA; R: GCAACCGGGCGATACGCCGTG	0–6 (7)	1 (2)	1 (2.4)	0 (0)	0 (0)	2 (2)	0.33 (0.15–0.50)	0.76 (0.65–0.87)	0.56 (0.42–0.70)
BcenM03_49bp_165bp_1U	49	315 (4)	165 (1)	315 (4)	F: GATGACGATCGGCGCGATAAT; R: ACGTCCATTCGCCCGCGATGA	1–8 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.73 (0.65–0.80)	0.66 (0.50–0.82)	0.73 (0.69–0.78)
BcenM04_49bp_229bp_3U	49	138 (1)	229 (3)	285 (4)	F: GTACTCGACGGGTTTGATCGT; R: TCATTTGGGCCGCCGGTTTT	0–4 (5)	2 (4)	0 (0)	0 (0)	0 (0)	2 (2)	0.11 (0.00–0.24)	0.66 (0.54–0.78)	0.61 (0.47–0.73)
BcenM05_49bp_508bp_2U	49	493 (2)	508 (2)	553 (3)	F: AAATACGCGTAGTAGATGCTGTG; R: TTCGACTTACGTATATCGCCGG	2–5 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0.39 (0.21–0.57)	0.43 (0.33–0.52)
BcenM06_49bp_438bp_1U	49	599 (4)	438 (1)	603 (4)	F: CGCTCGACTTCGCGCGCGATCA; R: ACGACGAACTTGCGCTCGTCGA	1–4 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0.54 (0.43–0.65)	0.55 (0.47–0.66)
BcenM07_17bp_140bp_2U	17	139 (2)	140 (2)	156 (3)	F: TTCACGCGTGATGCGGCGGGCG; R: TGACGGTCGCCCGCACGCGCA	0–4 (4)	0 (0)	1 (2.4)	8 (100)	0 (0)	9 (9)	0	0.49 (0.33–0.65)	0.42 (0.31–0.53)
BcenM08_75bp_1023bp_9U	75	1173 (11)	1023 (9)	948 (8)	F: CCGGCCGTGCTGTTGCCGAG; R: CACGTCGTTGTCGACCGGGGTT	0–11 (7)	0 (0)	1 (2.4)	8 (100)	0 (0)	9 (9)	0.71 (0.66–0.76)	0.62 (0.48–0.75)	0.71 (0.66–0.77)
BcenM09_49bp_346bp_2U	49	396 (3)	346 (2)	446 (4)	F: GTGCGGGGCGACCAGAACACGCG; R: ATCTGGATCGGGATCGTGATGA	0–7 (6)	26 (53)	0 (0)	0 (0)	0 (0)	26 (26)	0.65 (0.56–0.75)	0.62 (0.49–0.74)	0.72 (0.69–0.76)
BcenM10_54bp_265bp_3U	54	307 (4)	265 (3)	340 (5)	F: TGGCGCGCGGGCTGAGGCAT; R: CGCCGCCCGTGCATGACACGC	0–6 (6)	0 (0)	3 (7.3)	4 (50)	2 (100)	9 (9)	0.05 (0.00–0.16)	0.54 (0.33–0.75)	0.59 (0.48–0.70)

[a] F, forward; R, reverse. Annealing temperature, 60°C.

[b] 95% CI, 95% confidence interval. The global HGDI values (95% CI) were 0.96 (0.93 to 0.99) for IIIA, 0.97 (0.94 to 1.00) for IIIB, and 0.98 (0.97 to 0.99) total.

strains J2315 and AU1054 (of a known expected size), and two DNA size markers, i.e., 50-bp DNA ladder and pUC mix marker 8 (Fermentas, Villebon-sur-Yvette, France), were included in each run to allow size assignment.

Performance criteria for evaluation of MLVA typing. MLVA was evaluated according to the Guidelines for the validation and application of typing methods for use in bacterial epidemiology (19).

The interlaboratory reproducibility of MLVA was tested on 11 of the 25 reference strains, composed of seven clinical strains (LMG16656, LMG18827, LMG18826, LMG24506, LMG6981, LMG18828, and LMG16659) and four environmental strains (LMG19232, LMG19233, LMG19243, and LMG19248). MLVA was performed at the University Paris Sud, Orsay laboratory, and in the Observatoire *B. cepacia* laboratory, Toulouse, France, and the results were compared.

Typeability (T) was assessed for each of the 10 VNTR loci tested (Table 2). Strains for which two or more VNTRs were not amplified were further submitted to *recA* sequencing in order to ascertain that their initial identification as *B. cenocepacia* by IIIA- or IIIB-specific PCR positivity was correct.

In vivo longitudinal stability (LS) was assessed by testing the longitudinal stability panel (see Table S1 in the supplemental material). *In vivo* transversal stability (TS) was assessed by comparing the MLVA profiles of 25 outbreak-related strains belonging to the three genotypes involved in major outbreaks in France and referred to as genotypes 2, 3, and 11 in a previous study (20) (Table 1).

Epidemiological concordance (E) was assessed by testing 39 strains, which included the abovementioned 25 outbreak-related clinical strains, six epidemiologically related strains involved in cross-transmission, four strains belonging to the ET-12 lineage, and four environmental reference strains of *B. cenocepacia* IIIC belonging to the same sequence type (ST-118).

Discriminatory power was evaluated using a panel of 60 epidemiologically unrelated clinical strains belonging to the IIIA (36 strains) and IIIB (24 strains) subgroups, which included 10 reference strains and one strain of each epidemic clone (see Table S2 in the supplemental material). The Hunter-Gaston discriminatory index (HGDI) and 95% confidence interval were calculated using the BioNumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).

The MLVA typing results were compared to the results obtained by PCR ribotyping (21) routinely used for epidemiological screening in the Observatoire *B. cepacia* laboratory and PFGE, performed as previously described (20).

Finally, the unweighted-pair group method using average linkages (UPGMA) was used for clustering analysis and dendrogram generation (BioNumerics).

***recA* sequencing and MLST.** *recA* sequencing was performed on nontypeable strains according to Mahenthiralingam et al. (18), and the sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/).

A selection of strains was subjected to MLST using the scheme proposed by Baldwin et al. (22). Amplification was performed in a reaction volume of 50 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 1 M betaine, 1.5 U of Taq DNA polymerase, 200 μM each deoxynucleoside triphosphate, 0.4 μM each primer, and 4 μl of DNA template. Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). DNA concentration and purity were assessed using spectrophotometric analysis (BioPhotometer; Eppendorf, Le Pecq, France) and DNA concentrations adjusted to 10 ng/μl. The sequencing reactions were performed by the genomic facility GeT-Purpan of the Genomic and Transcriptomic Platform, Génopole Toulouse-Midi-Pyrénées, using the ABI Prism BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the 3130XL genetic analyzer (Applied Biosystems). The *B. cepacia* complex MLST website (http://pubmlst.org/bcc/) developed by Keith Jolley and located at the University of Oxford, Oxford, United Kingdom (23) was used for the MLST analysis.

IS*1363*-specific PCR. The primers P1 (5′-GCTTAATAGGATGGTCAG-3′) and P2 (5′-TCCATGACCACCGTACAACTC-3′) were those described by Liu et al. (8). PCR was performed in a reaction volume of 25 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, 200 μM each deoxynucleoside triphosphate, 0.6 μM each primer, and 4 μl of DNA template. DNA from strain AU1054 was used as a positive control. Amplification was carried out under the following conditions: initial denaturation for 5 min at 95°C, 30 cycles of 45 s at 95°C, 45 s at 55°C, and 45 s at 72°C, with a final extension step of 10 min at 72°C. The PCR products were separated in 1.5% (wt/vol) agarose gels (Eurobio) in TBE buffer.

RESULTS

VNTR identification and interlaboratory reproducibility. Fourteen VNTRs were identified by a genome comparison of three available sequenced strains. Ten were subsequently retained on the basis of a minimum repeat size of 15 bp and more than one allele within the 11 reference strains constituting the test panel for reproducibility (Table 2). The MLVA results were identical in both laboratories for the 11 strains.

Typeability. Amplification failures were observed at two, three, or four VNTRs in 18 of the 109 strains tested: 8/18 were environmental *B. cenocepacia* IIIC strains, which were all negative for at least BcenM07 and BcenM08; five were IIIA *recA*-based specific PCR positive, and subsequent *recA* sequencing identified four of them as *B. contaminans* (with at least BcenM07, BcenM08, and BcenM10 being negative) and the last one as *Burkholderia cenocepacia* IIIA; last, five were IIIB *recA*-based specific PCR positive, and subsequent *recA* sequencing identified them as belonging to the BCC6 group described by Dalmastri et al. (24). The nine BCC6 group and *B. contaminans* strains were excluded from further analysis, which was consequently based on 100 *B. cenocepacia* strains (Table 2). Individual marker typeability was found to be ≥0.98 for six markers (BcenM01 to BcenM06) and 0.91 for three markers (BcenM07, BcenM08, and BcenM10). Last, BcenM09 amplification failure was observed in 26 of the 49 *B. cenocepacia* IIIA strains (53%), in contrast with the full BcenM09 typeability of other subgroups. The global T values for the MLVA-10 scheme were 0.43 for *B. cenocepacia* IIIA strains and 0.80 for *B. cenocepacia* IIIB strains. The low global typeability of *B. cenocepacia* IIIA strains is partly due to the overrepresentation (11/49) of ST-32 strains, for which BcenM09 amplification was never obtained. In addition, BcenM09 was not amplified in three strains sharing the PCR ribotype CE and the PFGE type DE (ST-201). Thus, considering the absence of BcenM09 amplification as a signature for ST-32 and ST-201 strains, it is possible to evaluate BcenM09 typeability as 0.76 and the global typeability of *B. cenocepacia* IIIA strains as 0.69.

Allelic variation. The number of alleles of the different markers ranged from three to seven within the 100 tested strains (Table 2). Of note is that all the strains of *B. cenocepacia* IIIA tested in this study ($n = 49$) were monomorphic with regard to the number of repeat units of BcenM04 (three repeat units, with the exception of two amplification failures), BcenM05 (two repeat units), BcenM06 (one repeat unit), and BcenM07 (two repeat units), and they were almost monomorphic at BcenM10 (mostly three repeat units except for one strain). Figure 1 shows an example of an electrophoretic analysis of VNTR polymorphisms.

Longitudinal stability. The longitudinal stability panel (see Table S1 in the supplemental material) included 39 isolates from 14 patients. Nine patients were colonized with *B. cenocepacia* IIIA

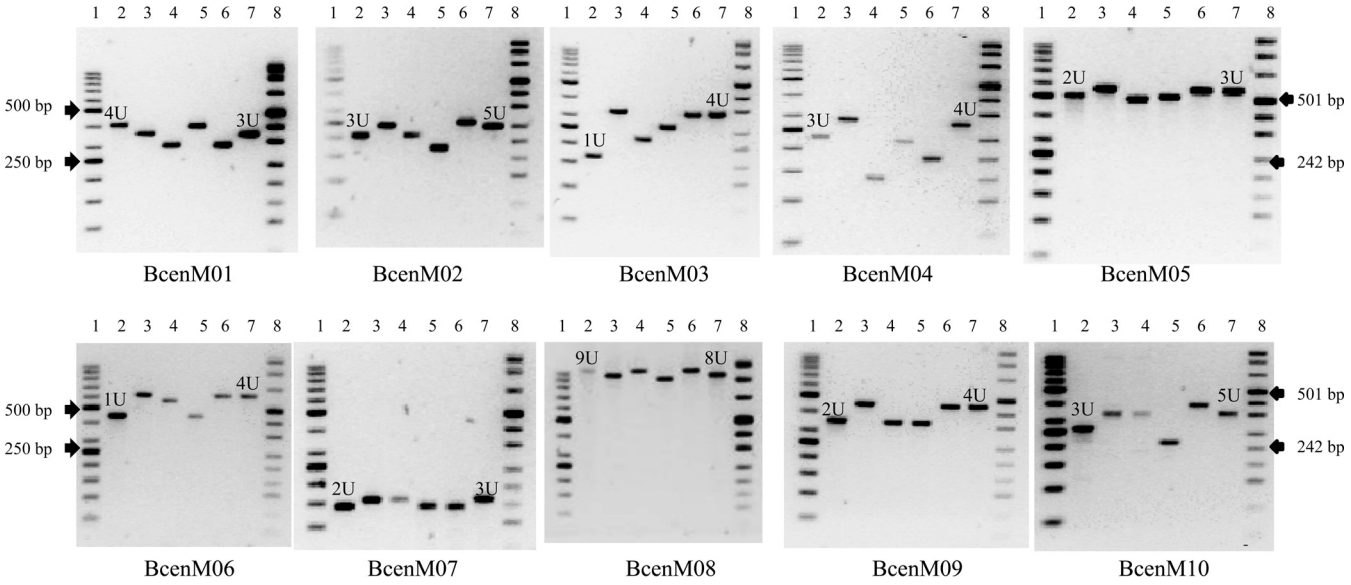


FIG 1 Electrophoretic analysis of the number of tandem repeats at each VNTR. Lane 1, 50-bp DNA ladder; lane 2, strain J2315 (LMG16656); lanes 3 to 6, four of the strains tested in the present study; lane 7, strain AU1054 (LMG24506); lane 8, pUC mix marker 8. The numbers of tandem repeats (U) are indicated for J2315 and AU1054 strains, used as controls. The arrows indicate fragment sizes of the DNA ladders.

and five with *B. cenocepacia* IIIB. The mean time between the first and the last isolate was 4.7 years (range, 1.1 to 10.2). In 12 of the 14 patients (86%), the MLVA profiles were identical over time. In two patients, a difference at BcenM08 was observed in one of the isolates tested, i.e., one amplification failure and a deletion of one repeat. Thus, an LS value of 1.00 was achieved for all markers except BcenM08 (LS, 0.90).

Transversal stability and epidemiological concordance. Twenty-five French outbreak-related strains, involving three epidemic lineages, were tested. All strains within each epidemic lineage harbored the same MLVA profile, with the exception of two amplification failures (at BcenM02 and BcenM10). Thus, a TS value of 1.00 was achieved for all markers. except BcenM02 and BcenM10 (TS, 0.96).

Two pairs of French epidemiologically related strains showed the same MLVA type, and within a third pair, the only difference was that BcenM04 failed to be amplified in one isolate. Similarly, four strains belonging to the *B. cenocepacia* IIIA ET-12 lineage and four ST-118 environmental *B. cenocepacia* IIIC strains were identical. In conclusion, epidemiological concordance assessed using 39 epidemiologically related strains showed an E value of 0.92 (Table 1).

Discriminatory power. The discriminatory power of the MLVA scheme was assessed using a panel of 60 epidemiologically unrelated strains (see Table S2 in the supplemental material). The HGDI values and 95% confidence intervals are reported in Table 2. An HGDI value of 0.98 was observed when the 10 VNTRs were combined. Five of the 10 markers (BcenM01, BcenM03, BcenM04, BcenM08, and BcenM09) were highly discriminating (HGDI, >0.6), whereas the remaining markers provided HGDI values of 0.42 to 0.59. A marked difference was observed between the IIIA and IIIB strains. Within the IIIA strains, only three markers (BcenM03, BcenM08, and BcenM09) provided HGDI values of >0.6, whereas five markers provided HGDI values of <0.3. However, the combined index was similar for both groups, reflecting the marker complementarities. The numbers of IIIC and IIID strains included in the present study were low (eight and two strains, respectively), and allelic variation was observed with only two markers (BcenM02 and BcenM05 and BcenM05 and BcenM08, respectively).

Comparison of MLVA and PFGE within *B. cenocepacia* IIIA and IIIB strains. PFGE results were available for 82 strains, which included 44 *B. cenocepacia* IIIA and 38 *B. cenocepacia* IIIB strains, and these were compared with the results of MLVA typing. Strains exhibiting contradictory PFGE and MLVA classifications were submitted to MLST. The 82 strains were distributed into 41 MLVA types and 47 PFGE types.

Fifty-three strains were classified into 12 shared MLVA types that included 2 to 10 strains, as well as 24 PFGE types. Of the 12 shared MLVA types, two (A5 and B2) included epidemiologically related strains only, three (A1, A3, and B1) included epidemiologically related and unrelated strains, six (A4, A12, A20, A18, B5, and B8) included epidemiologically unrelated strains only, and the last one (B4), had strains LMG14274, LMG14276, and LMG13011, which were recovered from CF patients in Belgium. The PFGE results were in agreement with the MLVA classifications within six of these 12 shared MLVA types, i.e., A12, A20, A5, B2, B4, and B5, which encompassed 22 strains. Conversely, several PFGE types were differentiated within the six remaining shared MLVA types consisting of 31 strains. The 25 strains belonging to MLVA types A1, B1, and A3 were distributed into three, five, and four PFGE types, respectively, whereas MLST analysis grouped the MLVA type A1 strains into ST-32, MLVA type B1 strains into ST-122, and MLVA type A3 strains into three very close STs (ST-276, ST-234, and ST-869, with two nucleotides difference). Two pairs of *B. cenocepacia* IIIA epidemiologically unrelated strains shared a PCR ribotype (FK and CU, respectively) and MLVA types (A4 and A18, respectively) but were classified into different PFGE types and different STs, mainly due to differences in the *gyrB* allele. Finally, two strains of *B. cenocepacia* IIIB classified into the B8

MLVA type were different by all the other techniques. Within the nine shared MLVA types that included epidemiologically unrelated strains, four match globally distributed STs (MLVA type A1/ST-32, MLVA type A3/ST-234, MLVA type A20/ST-241, and MLVA type B1/ST-122), and two match STs reported in another country (MLVA type A12/ST-201 and MLVA type A18/ST-665 in Italy and New Zealand, respectively) (http://pubmlst.org/bcc).

Twenty-nine strains were classified into 29 unique MLVA types and 24 PFGE types. Twenty of these 29 strains were classified into unique MLVA types and unique PFGE types. Three strains with unique MLVA types (21_17/MLVA type A24, 17_29/MLVA type B21, and 22_78/MLVA type A26) classified into shared PFGE types (BD, C, and HB, respectively) were part of the epidemiologically related panel and differed from the other strains harboring the same PFGE type by a "0" allele at one locus. Two pairs of epidemiologically unrelated strains (12_03 and 22_59, and 05_12 and 9_54) were classified into shared PFGE types (GO and C, respectively) and shared STs (844 and 279, respectively). One pair of epidemiologically unrelated strains (20_77 and 20_78) was classified into the same PFGE type (FY) and two different STs (ST-760 and ST-845, 8 nucleotides difference).

In conclusion, MLVA classification was in agreement with PFGE and/or MLST in 69 of 82 strains (84%).

Population analysis. UPGMA analysis showed that MLVA classification was in agreement with *recA* classification, with the exception of two IIIB strains. A cutoff of 85% was used to delineate clusters, corresponding to a maximum of one difference among the 10 loci (Fig. 2).

Four clusters designated IIIB-C1 to -C4 were delineated within *B. cenocepacia* IIIB strains, which included 26 of the 41 tested strains. MLVA clusterization within the *B. cenocepacia* IIIB isolates fit with epidemiological and/or genetic relatedness: cluster IIIB-C1 consisted of Belgian CF strains, cluster IIIB-C2 of ST-279 strains (French epidemic type 11, reported only in France to-date), cluster IIIB-C3 of ST-122 strains, including the PHDC North American epidemic strain and outbreak-related CF strains (French epidemic type 2), and cluster IIIB-C4 of epidemiologically unrelated strains (two CF and one non-CF), which exhibited the same PFGE type. Following the identification of French strains harboring the same MLVA type as strain AU1054/LMG24506 (PHDC *B. cenocepacia* IIIB lineage), characterized by the presence of IS*1363*, an IS*1363*-specific PCR was performed in *B. cenocepacia* IIIB-PCR-positive strains. Strain OBC08_30, which was classified in cluster IIIB-C3 but belongs to ST-37 (10 nucleotide and 3 allele differences with ST-122) was IS*1363* positive. Of note is that five *B. cenocepacia* IIIB strains were found to be IS*1363* positive outside the IIIB-C3 cluster.

Six clusters designated C1 to C6 were delineated within the *B. cenocepacia* IIIA strains, which included 35 of the 49 strains tested. MLVA clusterization within the *B. cenocepacia* IIIA isolates fit with epidemiological and/or genetic relatedness for five of the six clusters: cluster IIIA-C1 was composed of ET-12 strains (and of a French CF strain), cluster IIIA-C2 and IIIA-C5 of groups of strains belonging to very close STs (one to two nucleotides difference), IIIA-C4 of strains classified into the same PCR ribotype and PFGE type, and IIIA-C6 of ST-32 strains (French epidemic type 3). On the contrary, cluster IIIA-C3 was heterogeneous (six strains, four PCR ribotypes, five PFGE types, and five markedly different STs).

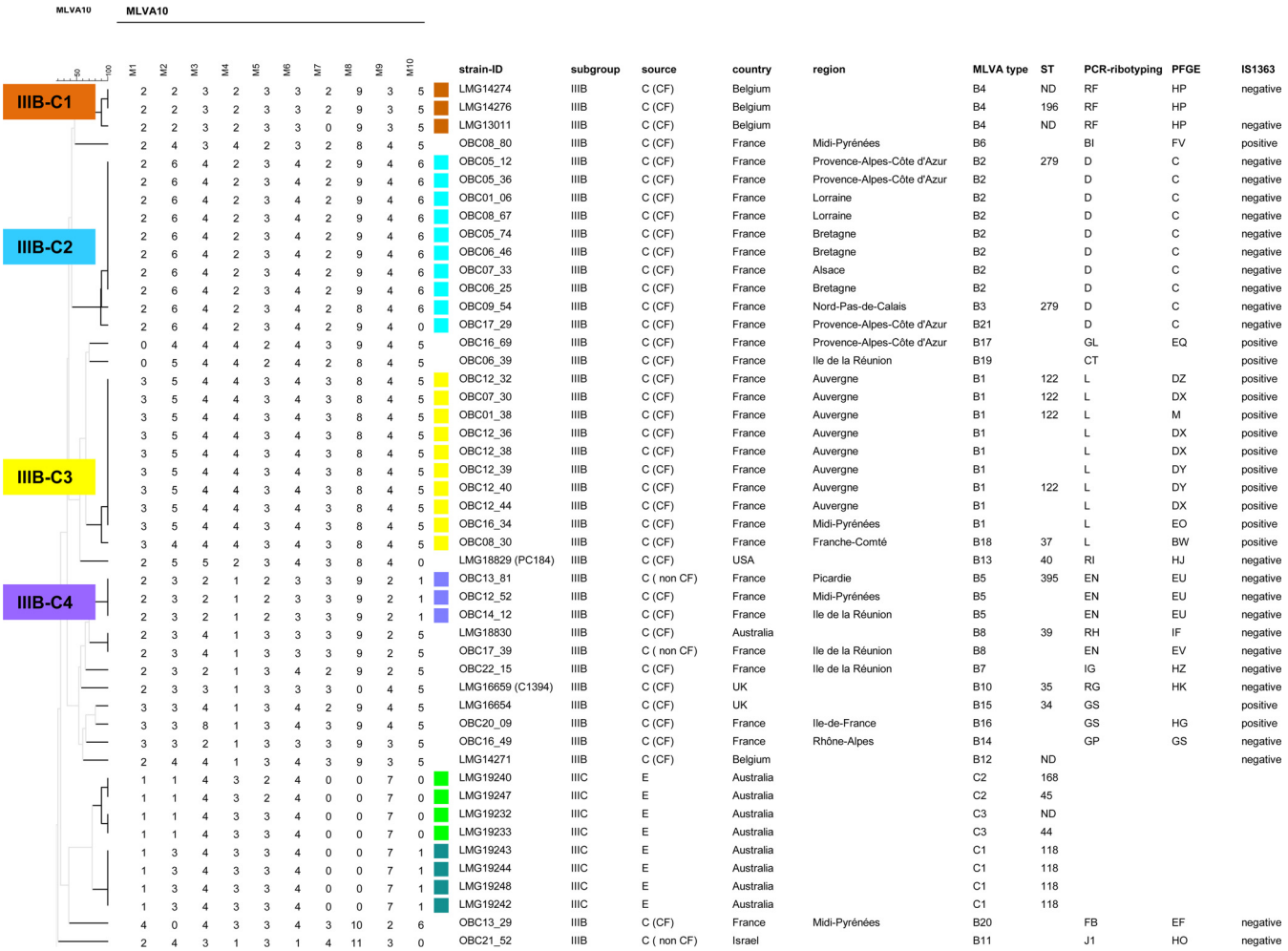
Lowering the cutoff to 58% classified nearly all the *B. cenocepacia* IIIA strains into the same group but delineated five groups within the *B. cenocepacia* IIIB strains.

DISCUSSION

BCC organisms are recognized as important pathogens in CF. Due to their ability to spread from patient to patient, they have been involved in large outbreaks. While PCR-based methods and PFGE have successfully been applied to local epidemiology, large-scale analyses require more portable methods, such as MLST or MLVA. An MLST scheme based on seven housekeeping genes has been developed and applied to global epidemiological analyses (22, 25). Also, the *recA* gene included in the MLST scheme allows the discrimination of species within the BCC. MLVA is also a transferable typing method, which is increasingly used to trace bacterial epidemiology. In the present study, we established an MLVA-10 scheme for the typing of *B. cenocepacia* strains, which together with *B. multivorans* predominates in CF and is associated with highly transmissible lineages, such as the so-called ET-12, ST-32 (*B. cenocepacia* IIIA), Midwest, and PHDC (*B. cenocepacia* IIIB) lineages. Within the selected markers, the size of the repeat unit varied from 15 to 75 bp, which allows for allele assignment by means of simple agarose gel electrophoresis. The performance of MLVA-10 was evaluated on a large representative collection of epidemiologically related and unrelated national and international strains.

The selected VNTRs appear to be specific for *B. cenocepacia*, as *B. contaminans* and the BCC6 group strains failed to be amplified at two to four loci when using the proposed PCR primer pairs. An examination of the sequenced genomes confirmed that the VNTRs cannot be amplified *in silico* in other members of the BCC. Moreover, these loci are not equally spread within the subgroups of the *B. cenocepacia* species. BcenM09 failed to amplify in more than half of the *B. cenocepacia* IIIA strains tested, whereas it was amplified in all the *B. cenocepacia* IIIB, IIIC, and IIID strains tested. BcenM07 and BcenM08 failed to amplify in the eight *B. cenocepacia* IIIC strains tested, whereas they were amplified in all but one of the 92 strains belonging to other subgroups. Nevertheless, the absence of BcenM09 may be considered a marker for some *B. cenocepacia* lineages (ST-32 and ST-201), and the fact that *B. cenocepacia* IIIC strains are not fully typeable with the proposed scheme is not expected to impact the performance of MLVA for the typing of clinical strains, since subgroup IIIC has until now been recovered from the natural environment only.

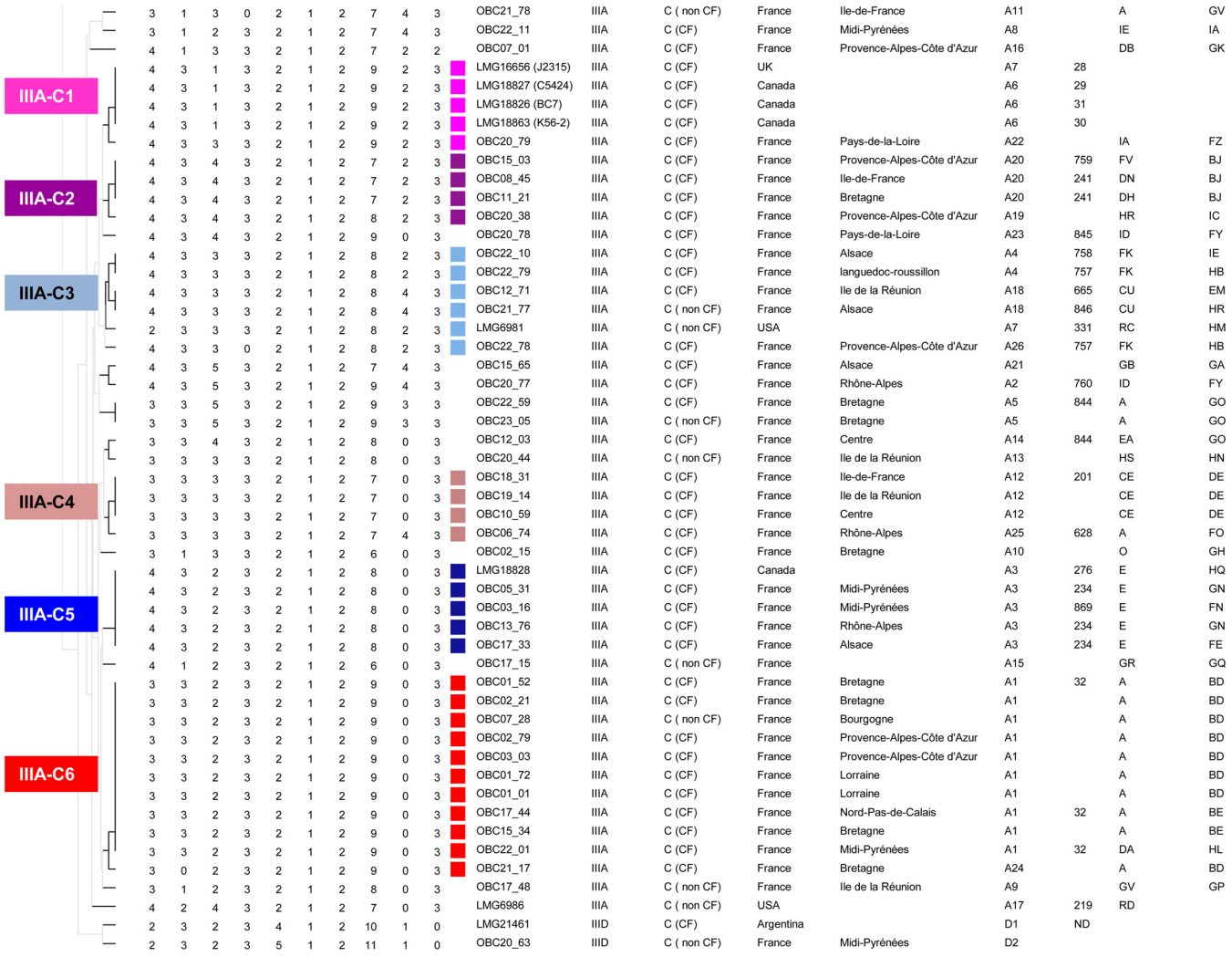
Stability is of the utmost importance in BCC epidemiology, since most colonizations are chronic and since patient-to-patient spread may be observed over years. We checked the stabilities of the selected VNTR loci in 14 patients by testing up to four successive isolates per patient. The MLVA patterns were stable over time in 13 patients (92.8%), and the variation observed in one patient concerned a single VNTR (BcenM08, with a loss of one repeat unit). Comparatively, the longitudinal stability of the MLST results for 41 BCC isolates from 20 patients showed no change in 15 patients (75%), strain replacement in one patient, and changes due to recombination events in four patients (20%) colonized with *B. multivorans* (one patient), *B. vietnamiensis* (two patients), or *B. cenocepacia* IIIA (one patient) (26). If this trend was confirmed by larger investigations, then one might speculate that allelic differences at VNTR loci in the BCC may prevent recombination, and it would explain the stability of MLVA compared to that of the MLST profiles. In order to validate the use of MLVA for



strain-ID	subgroup	source	country	region	MLVA type	ST	PCR-ribotyping	PFGE	IS1363
LMG14274	IIIB	C (CF)	Belgium		B4	ND	RF	HP	negative
LMG14276	IIIB	C (CF)	Belgium		B4	196	RF	HP	
LMG13011	IIIB	C (CF)	Belgium		B4	ND	RF	HP	negative
OBC08_80	IIIB	C (CF)	France	Midi-Pyrénées	B6		BI	FV	positive
OBC05_12	IIIB	C (CF)	France	Provence-Alpes-Côte d'Azur	B2	279	D	C	negative
OBC05_36	IIIB	C (CF)	France	Provence-Alpes-Côte d'Azur	B2		D	C	negative
OBC01_06	IIIB	C (CF)	France	Lorraine	B2		D	C	negative
OBC08_67	IIIB	C (CF)	France	Lorraine	B2		D	C	negative
OBC05_74	IIIB	C (CF)	France	Bretagne	B2		D	C	negative
OBC06_46	IIIB	C (CF)	France	Bretagne	B2		D	C	negative
OBC07_33	IIIB	C (CF)	France	Alsace	B2		D	C	negative
OBC06_25	IIIB	C (CF)	France	Bretagne	B2		D	C	negative
OBC09_54	IIIB	C (CF)	France	Nord-Pas-de-Calais	B3	279	D	C	negative
OBC17_29	IIIB	C (CF)	France	Provence-Alpes-Côte d'Azur	B21		D	C	negative
OBC16_69	IIIB	C (CF)	France	Provence-Alpes-Côte d'Azur	B17		GL	EQ	positive
OBC06_39	IIIB	C (CF)	France	Ile de la Réunion	B19		CT		positive
OBC12_32	IIIB	C (CF)	France	Auvergne	B1	122	L	DZ	positive
OBC07_30	IIIB	C (CF)	France	Auvergne	B1	122	L	DX	positive
OBC01_38	IIIB	C (CF)	France	Auvergne	B1	122	L	M	positive
OBC12_36	IIIB	C (CF)	France	Auvergne	B1		L	DX	positive
OBC12_38	IIIB	C (CF)	France	Auvergne	B1		L	DX	positive
OBC12_39	IIIB	C (CF)	France	Auvergne	B1		L	DY	positive
OBC12_40	IIIB	C (CF)	France	Auvergne	B1	122	L	DY	positive
OBC12_44	IIIB	C (CF)	France	Auvergne	B1		L	DX	positive
OBC16_34	IIIB	C (CF)	France	Midi-Pyrénées	B1		L	EO	positive
OBC08_30	IIIB	C (CF)	France	Franche-Comté	B18	37	L	BW	positive
LMG18829 (PC184)	IIIB	C (CF)	USA		B13	40	RI	HJ	negative
OBC13_81	IIIB	C (non CF)	France	Picardie	B5	395	EN	EU	negative
OBC12_52	IIIB	C (CF)	France	Midi-Pyrénées	B5		EN	EU	negative
OBC14_12	IIIB	C (CF)	France	Ile de la Réunion	B5		EN	EU	negative
LMG18830	IIIB	C (CF)	Australia		B8	39	RH	IF	negative
OBC17_39	IIIB	C (non CF)	France	Ile de la Réunion	B8		EN	EV	negative
OBC22_15	IIIB	C (CF)	France	Ile de la Réunion	B7		IG	HZ	negative
LMG16659 (C1394)	IIIB	C (CF)	UK		B10	35	RG	HK	negative
LMG16654	IIIB	C (CF)	UK		B15	34	GS		positive
OBC20_09	IIIB	C (CF)	France	Ile-de-France	B16		GS	HG	positive
OBC16_49	IIIB	C (CF)	France	Rhône-Alpes	B14		GP	GS	negative
LMG14271	IIIB	C (CF)	Belgium		B12	ND			negative
LMG19240	IIIC	E	Australia		C2	168			
LMG19247	IIIC	E	Australia		C2	45			
LMG19232	IIIC	E	Australia		C3	ND			
LMG19233	IIIC	E	Australia		C3	44			
LMG19243	IIIC	E	Australia		C1	118			
LMG19244	IIIC	E	Australia		C1	118			
LMG19248	IIIC	E	Australia		C1	118			
LMG19242	IIIC	E	Australia		C1	118			
OBC13_29	IIIB	C (CF)	France	Midi-Pyrénées	B20		FB	EF	negative
OBC21_52	IIIB	C (non CF)	Israel		B11		J1	HO	negative

FIG 2 MLVA-based UPGMA clustering analysis of the *B. cenocepacia* strains included in the present study (cutoff, 80%). Strains classified in the same cluster are indicated using colored squares. Under the source column, there are clinical (C) and environmental (E) strains. ID, identification; CF, cystic fibrosis.

identifying outbreaks, we tested multiple strains of the three epidemic lineages of *B. cenocepacia*, i.e., ST-32, ST-279, and ST-122, responsible for outbreaks in one (ST-122) or several (ST-32 and ST-279) French CF centers in the late 1980s to the early 1990s. We included strains from each of the CF centers, collected over periods of 11 to 22 years, and demonstrated the long-term transversal stability of the MLVA profiles, with the exception of two amplification failures. Of note is that a loss of one BcenM08 repeat unit was observed in one outbreak-unrelated ST-279 strain (OBC09_54). Thus, the marker BcenM08 might be slightly less stable than the other markers, since it also was the single marker to vary in one patient who was included in the longitudinal study. Whereas the PFGE types were identical within all the ST-279 strains tested, two, two, and five PFGE types were observed within the globally distributed lineage ST-32, ST 234, and ST-122 (PHDC) strains, respectively. PFGE pattern variability was previously reported by Drevinek and Mahenthiralingam (27) within the ST-32 Czech strains, and it was attributed to IS-mediated genomic rearrangements. Similar variability was reported by Campana et al. (28) within the Italian PHDC strains. It can be hypothesized that due to the presence of several copies of IS*1363* in PHDC strains (8), IS movements might also interfere with macrorestriction-based types within this lineage. Interestingly, we demonstrated the presence of IS*1363* in 16 of the 41 *B. cenocepacia* IIIB strains tested, six of which were different from the PHDC lineage. Lastly, the four tested ET-12 strains exhibited the same MLVA type, whereas they belong to four different STs identified within Canadian ET-12 strains. In contrast, all reported ET-12 strains in the United Kingdom were found to belong to ST-28 (29). Thus, the evolution rates and mechanisms within each major clonal complex seem different, with variable consequences according to the typing method, leading to difficulties in establishing a unique gold standard for genotyping. This is in agreement with a previous study using multilocus restriction typing (MLRT) (30), showing differences in population structures among the ET-12, PHDC, and Midwest lineages, which were attributed to various degrees of genetic recombination. Finally, the MLVA-10 signatures of other epidemic strains, i.e., LMG18829 (ST-40, Midwest clone) and LMG16659 (ST-35, Manchester epidemic clone), were shown to be specific within the collection tested. On the contrary, LMG18830 (ST-36, Sydney epidemic clone) and a non-CF strain from La Réunion Island were classified in the same MLVA type but into different PCR ribotypes, PFGE types, and MLST profiles. This might be attributed to homoplasy, i.e., evo-



3	1	3	0	2	1	2	7	4	3		OBC21_78	IIIA	C (non CF)	France	Ile-de-France	A11		A	GV
3	1	2	3	2	1	2	7	4	3		OBC22_11	IIIA	C (CF)	France	Midi-Pyrénées	A8		IE	IA
4	1	3	3	2	1	2	7	2	2		OBC07_01	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A16		DB	GK
4	3	1	3	2	1	2	9	2	3		LMG16656 (J2315)	IIIA	C (CF)	UK		A7	28		
4	3	1	3	2	1	2	9	2	3		LMG18827 (C5424)	IIIA	C (CF)	Canada		A6	29		
4	3	1	3	2	1	2	9	2	3		LMG18826 (BC7)	IIIA	C (CF)	Canada		A6	31		
4	3	1	3	2	1	2	9	2	3		LMG18863 (K56-2)	IIIA	C (CF)	Canada		A6	30		
4	3	3	3	2	1	2	9	2	3		OBC20_79	IIIA	C (CF)	France	Pays-de-la-Loire	A22		IA	FZ
4	3	4	3	2	1	2	7	2	3		OBC15_03	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A20	759	FV	BJ
4	3	4	3	2	1	2	7	2	3		OBC08_45	IIIA	C (CF)	France	Ile-de-France	A20	241	DN	BJ
4	3	4	3	2	1	2	7	2	3		OBC11_21	IIIA	C (CF)	France	Bretagne	A20	241	DH	BJ
4	3	4	3	2	1	2	8	2	3		OBC20_38	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A19		HR	IC
4	3	4	3	2	1	2	9	0	3		OBC20_78	IIIA	C (CF)	France	Pays-de-la-Loire	A23	845	ID	FY
4	3	3	3	2	1	2	8	2	3		OBC22_10	IIIA	C (CF)	France	Alsace	A4	758	FK	IE
4	3	3	3	2	1	2	8	2	3		OBC22_79	IIIA	C (CF)	France	languedoc-roussillon	A4	757	FK	HB
4	3	3	3	2	1	2	8	4	3		OBC12_71	IIIA	C (CF)	France	Ile de la Réunion	A18	665	CU	EM
4	3	3	3	2	1	2	8	4	3		OBC21_77	IIIA	C (non CF)	France	Alsace	A18	846	CU	HR
2	3	3	3	2	1	2	8	2	3		LMG6981	IIIA	C (non CF)	USA		A7	331	RC	HM
4	3	3	0	2	1	2	8	2	3		OBC22_78	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A26	757	FK	HB
4	3	5	3	2	1	2	7	4	3		OBC15_65	IIIA	C (CF)	France	Alsace	A21		GB	GA
4	3	5	3	2	1	2	9	4	3		OBC20_77	IIIA	C (CF)	France	Rhône-Alpes	A2	760	ID	FY
3	3	5	3	2	1	2	9	3	3		OBC22_59	IIIA	C (CF)	France	Bretagne	A5	844	A	GO
3	3	5	3	2	1	2	9	3	3		OBC23_05	IIIA	C (non CF)	France	Bretagne	A5		A	GO
3	3	4	3	2	1	2	8	0	3		OBC12_03	IIIA	C (CF)	France	Centre	A14	844	EA	GO
3	3	3	3	2	1	2	8	0	3		OBC20_44	IIIA	C (non CF)	France	Ile de la Réunion	A13		HS	HN
3	3	3	3	2	1	2	7	0	3		OBC18_31	IIIA	C (CF)	France	Ile-de-France	A12	201	CE	DE
3	3	3	3	2	1	2	7	0	3		OBC19_14	IIIA	C (CF)	France	Ile de la Réunion	A12		CE	DE
3	3	3	3	2	1	2	7	0	3		OBC10_59	IIIA	C (CF)	France	Centre	A12		CE	DE
3	3	3	3	2	1	2	7	4	3		OBC06_74	IIIA	C (CF)	France	Rhône-Alpes	A25	628	A	FO
3	1	3	3	2	1	2	6	0	3		OBC02_15	IIIA	C (CF)	France	Bretagne	A10		O	GH
4	3	2	3	2	1	2	8	0	3		LMG18828	IIIA	C (CF)	Canada		A3	276	E	HQ
4	3	2	3	2	1	2	8	0	3		OBC05_31	IIIA	C (CF)	France	Midi-Pyrénées	A3	234	E	GN
4	3	2	3	2	1	2	8	0	3		OBC03_16	IIIA	C (CF)	France	Midi-Pyrénées	A3	869	E	FN
4	3	2	3	2	1	2	8	0	3		OBC13_76	IIIA	C (CF)	France	Rhône-Alpes	A3	234	E	GN
4	3	2	3	2	1	2	8	0	3		OBC17_33	IIIA	C (CF)	France	Alsace	A3	234	E	FE
4	1	2	3	2	1	2	6	0	3		OBC17_15	IIIA	C (non CF)	France		A15		GR	GQ
3	3	2	3	2	1	2	9	0	3		OBC01_52	IIIA	C (CF)	France	Bretagne	A1	32	A	BD
3	3	2	3	2	1	2	9	0	3		OBC02_21	IIIA	C (CF)	France	Bretagne	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC07_28	IIIA	C (non CF)	France	Bourgogne	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC02_79	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC03_03	IIIA	C (CF)	France	Provence-Alpes-Côte d'Azur	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC01_72	IIIA	C (CF)	France	Lorraine	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC01_01	IIIA	C (CF)	France	Lorraine	A1		A	BD
3	3	2	3	2	1	2	9	0	3		OBC17_44	IIIA	C (CF)	France	Nord-Pas-de-Calais	A1	32	A	BE
3	3	2	3	2	1	2	9	0	3		OBC15_34	IIIA	C (CF)	France	Bretagne	A1		A	BE
3	3	2	3	2	1	2	9	0	3		OBC22_01	IIIA	C (CF)	France	Midi-Pyrénées	A1	32	DA	HL
3	0	2	3	2	1	2	9	0	3		OBC21_17	IIIA	C (CF)	France	Bretagne	A24		A	BD
3	1	2	3	2	1	2	8	0	3		OBC17_48	IIIA	C (non CF)	France	Ile de la Réunion	A9		GV	GP
4	2	4	3	2	1	2	7	0	3		LMG6986	IIIA	C (non CF)	USA		A17	219	RD	
2	3	2	3	4	1	2	10	1	0		LMG21461	IIID	C (CF)	Argentina		D1	ND		
2	3	2	3	5	1	2	11	1	0		OBC20_63	IIID	C (non CF)	France	Midi-Pyrénées	D2			

FIG 2 continued

lutionary convergence or reversion to an ancestral profile. Thus, in spite of one discordance, MLVA-10 seems a valuable tool for recognizing outbreaks and identifying widely distributed and/or epidemic lineages.

Within the 10 markers used, the number of alleles ranged from three to seven, and the diversity index (HGDI) for individual loci ranged from 0.42 to 0.73. The HGDI value for the MLVA-10 scheme was of 0.98, thus fulfilling the European Society of Clinical Microbiology and Infectious Diseases Study Group on Epidemiological Markers (ESGEM) criteria (>0.95) (19). Compared to PFGE within epidemiologically unrelated strains, the discriminatory power of MLVA-10 seems lower than that of PFGE, which was confirmed by MLST, although the reverse situation has also been observed. Concordance of MLVA with PFGE and/or MLST results was shown to be 84% within the collection tested.

MLVA-based population structure was in agreement with *recA* classification, with the exception of two IIIB strains, which might represent a specific subgroup. Heterogeneity was higher within the *B. cenocepacia* IIIB strains than within the *B. cenocepacia* IIIA strains, since three of the 10 loci were found to be homogeneous within the *B. cenocepacia* IIIA strains, which were nearly all classified in a unique group using a cluster delineation cutoff of 58%. This may be due to a lower discriminatory power of the scheme used within IIIA strains, but it is in agreement with an MLST-based population structure. Using the matches of five out of seven loci, 198 of the 212 *B. cenocepacia* IIIA strains included in the MLST database are brought together in a large clonal complex, with ST-234 as a potential ancestral type. In contrast, the 155 *B. cenocepacia* IIIB strains are distributed into 10 clonal complexes and 81 singletons (http://pubmlst.org/bcc/).

In conclusion, due to the high genomic plasticity of *B. cepacia* complex organisms, recombination events or IS movements may interfere with genotyping methods and mask epidemiological relatedness. The MLVA-10 scheme developed in the present study was shown to be an appropriate tool to detect epidemic strains of *B. cenocepacia*, due to the high stability of MLVA profiles, the satisfactory discriminatory power, and the portability of the method, allowing the establishment of a Web-based database (http://mlva.u-psud.fr) (14). We suggest the use of MLVA as a first-line typing method for *B. cenocepacia* and further analyses of the representative MLVA profiles by means of MLST. This approach would be cost-effective, since in our laboratory, the re-

agents and sequencing costs for the characterization of one strain by means of *recA* sequence for species identification plus MLVA-10 were 36 euros compared to 112 euros for MLST analysis, with comparable hands-on time. Besides, the MLVA-10 scheme could easily be automated using fluorescent primers and a single multiplex PCR (31). Capillary electrophoresis would allow automatic size measurement of the PCR products and thus increase the accuracy of repeat number determination, as described for other bacterial species (31–33). Furthermore, some potentially informative VNTR markers that were ruled out from the present scheme due to short repeats (<15 bp) could be included to constitute a second panel with high discriminatory power, as has been proposed for other bacteria (34). Nevertheless, in contrast with MLST, which has the advantage of being usable for the whole BCC, species-specific MLVA schemes have to be developed, and the establishment of a *B. multivorans* MLVA scheme is in progress.

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